# Growth and photosynthetic response of two larches exposed to $O_3$ mixing ratios ranging from preindustrial to near future

T. SUGAI[*], D.-G. KAM[*], E. AGATHOKLEOUS[*,**,+], M. WATANABE[*,***], K. KITA[#], and T. KOIKE[*,+]

*Silviculture and Forest Ecological Studies, Hokkaido University, Sapporo, Hokkaido 060-8689, Japan[*]*
*Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), 7 Hitsujigaoka, Sapporo, Hokkaido, 062-8516, Japan[**]*
*Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan[***]*
*Forestry Research Institute, Hokkaido Research Organization (HRO), Bibai, Hokkaido 079-0166, Japan[#]*

## Abstract

In this study, we questioned whether ground-level ozone ($O_3$) induces hormesis in Japanese larch (*Larix kaempferi*) and its hybrid $F_1$ (*L. gmelinii* var. *japonica* × *L. kaempferi*). In order to answer the question, we exposed seedlings of both taxa to four $O_3$ treatments [ranging from ≈10 to 60 nmol($O_3$) mol$^{-1}$] in open-top chambers for two consecutive growing seasons. We found a hormetic response in maximum photosynthetic rate ($P_{Nmax}$) at 1700 μmol($CO_2$) mol$^{-1}$ and maximum rates of carboxylation ($V_{cmax}$) and electron transport ($J_{max}$) in both larches. Stimulation of $P_{Nmax}$, $V_{cmax}$, and $J_{max}$ did not lead to suppressed plant productivity in Japanese larch, which followed a stress-tolerant strategy, but it did lead to suppressed plant productivity in hybrid larch which followed a competitive strategy. These findings are the first to suggest that stimulation of physiological functions by low $O_3$ exposures may have negative consequences for larch reproduction.

*Additional key words*: biphasic; competition; dose-response, homeostasis; hormesis; plasticity.

## Introduction

Hormesis is a biological phenomenon occurring in exposure-response studies, where low exposures to an agent stimulate the performance of an organism and high exposures inhibit its performance, therefore generating a biphasic exposure-response relationship (Calabrese 2015). Hormesis can be observed for biological and physiological processes in a cell or an organism, and has received increasing attention (Cedergreen *et al.* 2007, Calabrese 2013, 2015). Hormesis can be generalized as to stressors and organisms (*e.g.*, plants, animals, bacteria), both qualitative and quantitative (Calabrese 2013, 2014).

Larch is distributed broadly in the northeast Eurasian continent and northern America where permafrost occurs (Gower and Richards 1990, Osawa *et al.* 2010). Yet, larch, contributes significantly to fixing $CO_2$ in Eurasia and is promising for man-made forests (Ryu *et al.* 2009). Japanese larch [*L. kaempferi* (Lamb.) Carr.] has been widely planted in northeast Asia and partly introduced to central Europe from the 1930s (Matyssek and Schulze 1987, Ryu *et al.* 2009) due to its high growth rate, survival in xeric infertile soil condition, *etc.* (Qu 2016). To overcome some weak points (shoot blight disease and vole grazing), a hybrid larch $F_1$ (*L. gmelinii* var. *japonica* × *L. kaempferi*) has been developed and has many advantages: higher initial growth rate, resistance to biological stresses, and high specific gravity of a stem (Kita *et al.* 2009, Ryu *et al.* 2009). The high growth rate in larches may contribute to greater $CO_2$ fixation but also to higher uptake of gaseous pollutant (*e.g.*, Šesták 1985).

Ground-level $O_3$ concentrations have significantly increased over the past decades, especially in northeast Eurasian region (Akimoto *et al.* 2015, Feng *et al.* 2015, Verstraeten *et al.* 2015). Further increase has been predicted in the future (Ainsworth *et al.* 2012, Chatani *et al.* 2014). Overall, $O_3$ mixing ratios in the Northern Hemisphere are nowadays doubled as compared to the preindustrial period ones (Hartmann *et al.* 2013). Although precursor substances are decreasing in some areas or regions, $O_3$ concentrations remain at high mixing ratios due to transport of $O_3$ or $O_3$ precursor molecules at regional or trans-boundary level (Akimoto *et al.* 2015, Kalabokas *et al.* 2015, Sicard *et al.* 2016).

Ozone is a strong oxidant that severely affects vegetation (Paoletti *et al.* 2006, Agathokleous *et al.* 2016a, 2017). Ozone enters plant tissues *via* stomata and generates the production of reactive oxygen species (ROS); thus, forcing plants to undergo a stress-reponse process which often leads to suppressed photosynthetic capacity due to degradation of chlorophyll (Chl) and enzymes, and inhibition of growth and biomass accumulation (*e.g.*, Ainsworth *et al.* 2012, Agathokleous *et al.* 2016a). However, the plant response to $O_3$ varies among species (Yamaguchi *et al.* 2011, Koike *et al.* 2013, Feng *et al.* 2015) due to variability in stomatal response, carbon allocation patterns, and defense ability to detoxify radical oxygen species (Matyssek *et al.* 2007, Paoletti *et al.* 2008, Manninen *et al.* 2009).

Although the effects of $O_3$ on plants have been extensively studied over the past decades (Feng *et al.* 2015, Agathokleous *et al.* 2016a), the hypothesis that $O_3$ induces

hormesis in plants, *i.e.*, potential biphasic response in the full exposure-response continuum, has never been tested. Only a few studies provided evidence suggesting that low $O_3$ exposures may have stimulatory effects on plants (Eamus *et al.* 1990, Flowers *et al.* 2007, Vázquez-Ybarra *et al.* 2015). Whether $O_3$ induces hormesis, it requires further examination, especially, because the physiological response of plants to $O_3$ is influenced by various factors (*e.g.*, Ainsworth and Long 2005, Grantz *et al.* 2006).

The aim of this study was to test if chronic $O_3$ exposure induces hormetic responses in Japanese larch and its hybrid, which differ in their competitive capacity, and if there are differences in their responses. For this purpose, seedlings of the two larches were exposed to four $O_3$ treatments [ranging from ≈10 to 60 nmol($O_3$) mol$^{-1}$] for two consecutive growing seasons. The two taxa were selected because of prior evidence for their response to $O_3$ (Koike *et al.* 2012, Gyu *et al.* 2015, Agathokleous *et al.* 2017). Based on these prior studies, we hypothesized that elevated $O_3$ exposures may inhibit the growth over time. We further predicted that low $O_3$ [≈30 nmol($O_3$) mol$^{-1}$], which widely occurs in the Northern Hemisphere nowadays, may cause stimulatory effects on larches, compared to exposures similar to preindustrial concentrations [≈10 nmol($O_3$) mol$^{-1}$]. We discussed plausible understanding of $O_3$-induced hormesis in photosynthesis of larches in relation to potential competitive capacity for light and space because stress effects are driven by competition or tolerance, according to the primary strategies of plants (Grime 1977).

## Materials and methods

**Experimental site**: The experiment was conducted in the open-top chambers (OTCs) of Hokkaido University, located at Sapporo Experimental Forest of Hokkaido University in northern Japan (43°04' N, 141°20' E, 15 m a. s. l.). The experimental site was selected because of low background $O_3$ mixing ratios (Hoshika *et al.* 2013) compared to elevated $O_3$ mixing ratios commonly occurring in the Northern Hemisphere nowadays (*e.g.*, Feng *et al.* 2015, Sicard *et al.* 2016). The main meteorological conditions at the experimental area can be found in Agathokleous *et al.* (2016c, 2017).

**Plant material**: Two-year-old seedlings of Japanese larch (*L. kaempferi*) and its hybrid larch $F_1$ (*L. gmelinii* var. *japonica* × *L. kaempferi*) were directly planted in the ground of OTCs (brown forest soil, classified as Dystric Cambisols) in middle of May 2013.

The initial stem diameter of seedlings was 2.30 ± 0.04 mm for Japanese larch (JL) and 2.11 ± 0.04 mm for hybrid larch (HL), and the seedling height was 13.81 ± 0.24 and 17.94 ± 0.47 cm for JL and HL, respectively. After planting, 200 ml of liquid balanced fertilizer (1:1000, v/v, N:P:K = 6:10:5, *Hyponex Japan Corp., Ltd.,* Japan)

was applied to each seedling as soil drench.

**Experimental design**: In this experiment, four $O_3$ treatments were used: (a) a charcoal-filtered air treatment (CF) to imitate the preindustrial mixing ratio; (b) a nonfiltered air treatment (NF) representing low $O_3$ mixing ratios which occur nowadays; (c) a nonfiltered air enriched with additional $O_3$ to reach a target $O_3$ mixing ratio of 40 nmol($O_3$) mol$^{-1}$ (NF40); and (d) a nonfiltered air enriched with additional $O_3$ to reach a target $O_3$ mixing ratio of 60 nmol($O_3$) mol$^{-1}$ (NF60) simulating future elevated $O_3$ mixing ratios. The NF60 was set as environmental standard value for the photochemical oxidant of Japan (Ministry of the Environment in Japan 1996; http://www.env.go.jp/kijun/taiki.html). Each $O_3$ treatment was replicated four times, making a total of sixteen OTCs with four seedlings per larch variety in each OTC (128 seedlings in total), on a split-plot completely randomized design. The size of each OTC was 1.2 × 1.2 × 1.2 m in the first growing season and then extended to 2.2 m early in the second growing season due to an increased plant size. Each OTC was constructed as a steel frame (shaping an empty cube) which was perimetrically surrounded by a

polyvinyl chloride film (*Noh-bi*, Japan) with an 88% sunlight transmittance (blocking only UVB). For the CF treatment, charcoal-filtered air was added in the OTCs, whereas for the NF treatment ambient air was added to the OTCs. For the NF40 and NF60 treatments, additional O₃ was added to reach the target mixing ratio using a generator (*Model PZ-1 C, Kofloc,* Japan) which employs the pressure-swing-adsorption method for condensing the concentration of oxygen gas and using it as an O₃ gas material. This O₃ generation method produces just a tiny amount of NOx gas and is environmentally clean. A proportional-integrative-differential control algorithm was used to maintain the desired O₃ mixing ratio. The targeted O₃ mixing ratio was regulated by an O₃ monitor (*2B Tech*, NZ) and *Ebara* O₃-monitoring system (*Ebara,* Japan). The OTCs system was operated by *DALTON Co. Ltd.* (*Hokkaido Branch*, Japan). The O₃ treatment lasted from 17 June to 29 October 2013 and from 18 June to 15 September 2014, during the daylight hours (06:00–18:00 h, Japan Standard Time). The achieved O₃ mixing ratio and the values of mixing ratios accumulated over the threshold of 40 nmol(O₃) mol⁻¹ (AOT40, Fuhrer *et al.* 1997) for the four O₃ treatments over the treatment periods of the years 2013 and 2014 were:

| Treatment | O₃ mixing ratio [nmol(O₃) mol⁻¹] | | AOT40 [µmol(O₃) mol⁻¹ h] | |
|---|---|---|---|---|
| | 2013 | 2014 | 2013 | 2014 |
| CF | 12.2 ± 0.56 | 12.4 ± 0.35 | 0.827 | 0.846 |
| NF | 31.7 ± 1.53 | 29.9 ± 1.61 | 4.864 | 5.106 |
| NF40 | 40.1 ± 0.99 | 39.4 ± 1.03 | 10.671 | 8.776 |
| NF60 | 61.4 ± 1.68 | 62.5 ± 1.95 | 35.645 | 37.634 |

The temperature and light conditions were monitored using a *HOBO* pendant data logger (*UA-002-64, Onset Computer, Co*., USA) placed in the center of an OTC chamber, above the canopy of the plants; the OTC was in the middle of the OTC rows. One observation was recorded in a 5-min interval during August–October, in both years. The average daily temperature for the period of 06:00–18:00 was 20.53 ± 0.09 and 20.16 ± 0.14°C for the years 2013 and 2014, respectively, and the average daily PPFD was 24.15 ± 0.51 and 28.86 ± 0.56 mol(photon) m⁻² s⁻¹ for the years 2013 and 2014, respectively.

**Data collection**: Growth and dry matter production: The height and stem basal diameter (2 cm from stem base) of each seedling were measured on 17 June, 1 August, 6 September, and 6 October in 2013 and on 15 June and 20 September in 2014, using measuring tapes (1 mm gradient) and Vernier calipers (*Mitsutoyo,* Japan). The diameter was taken as the average of two crosswise measurements at the stem base. Since the sampling was not destructive, the relative growth rate (RGR) over a certain time interval was calculated for each plant using the classical approach (Hoffmann and Poorter 2002). The

equation was RGR = [ln(Pⱼ) − ln(Pᵢ)]/[tⱼ − tᵢ], where Pⱼ is the parameter value (height or diameter) at a certain time point, Pᵢ is the value at the preceding time point, and tⱼ and tᵢ is the time, when each measurement was conducted (in days). RGR is of particular importance when comparing growth between taxa and over a variety of environmental conditions (Whitehead and Myerscough 1962). The actual height gain was also calculated for each plant by subtracting the baseline height (17 June, 2013) from the final height (20 September, 2014), as an indicator of competition for light.

The 128 seedlings were harvested on 11 November 2014 by digging out the root system, and separated into foliage, branches, stem, and roots. The root system was gently washed with tap water to remove the soil particles. The samples were oven-dried at 70°C until constant dry mass and then weighed using a digital balance.

**Leaf gas exchange**: In early September 2014, gas-exchange measurements were taken from mature long-shoot needles of 122 seedlings. Measurements could not be taken from six seedlings (two hybrid larches and four Japanese larches) whose needles were severely injured by NF60 treatment. Sun-lit mature needles were randomly selected from the upper position of the crown. The gas-exchange rates were measured using an open gas-exchange system (*LI-6400, Li-Cor Inc.*, Lincoln, USA). The net photosynthetic rate ($P_N$), transpiration rate ($E$), and stomatal conductance ($g_s$) were determined at 380 µmol(CO₂) mol⁻¹, 60 ± 5% relative air humidity, and a PPFD of 1500 µmol(photon) m⁻² s⁻¹, as it has been previously described (Koike *et al.* 2012). The leaf temperature was maintained at 25°C. From the curve of net CO₂ assimilation rate *vs*. intercellular CO₂ concentration ($P_N/C_i$ curve), $P_{Nmax}$, $V_{cmax}$, and $J_{max}$ were calculated using an individual leaf photosynthetic model (Farquhar *et al.* 1980, Long and Bernacchi 2003). The water-use efficiency (WUE) was also calculated as the ratio of $P_N/E$.

After photosynthetic measurements, the measured needles were scanned and the measured needle area was calculated using the image analysis software *ImageJ* (*U. S. National Institutes of Health*, Bethesda, USA, Schneider *et al.* 2012).

**Leaf chlorophyll (Chl) and nutrients**: The needles used for gas-exchange measurements were collected for further analysis of Chl, C, and N contents. For Chl measurements, dimethyl sulfoxide (DMSO) was used to extract the Chl from the fresh needles. Then, the extracts were spectro-photometrically measured (*100-50*, *Hitachi*, Japan), and Chl contents were estimated as described by Shinano *et al.* (1996). For analysis of N and C content, the needles were dried at 70°C for at least 3 d until constant dry mass and then weighed. Nitrogen and C content in those needles were measured by a NC analyzer (*Elementar*, *VarioEL III*, Japan).

**Statistical analyzes**: For all the response variables, the data were averaged per experimental unit (block), thus, resulting in four values per $O_3$ treatment (one per OTC) per larch taxon for data analysis. For a better fitting to the Gaussian distribution, the data of each response variable were transformed with a Box-Cox transformation (Box and Cox 1964), as described by Agathokleous *et al.* (2016b). The data of each response variable (except height RGR and diameter RGR) were analyzed by general linear model (GLM) randomized by block, taxa, and $O_3$ were the predictors.

The height RGR and diameter RGR data were analyzed by repeated GLM models with between-subjects factors the taxa and $O_3$ and within-subjects factor the time which was carrying five levels. For significant factors with more than two levels, the single-step multiple comparison test of *Tukey*'s honest significance difference (HSD) was followed. The defined level of significance for all the statistical tests was $\alpha = 0.05$.

Simple linear regression analysis was conducted ($n = 32$) between pairs of Chl *a*, Chl *b* or Chl (*a*+*b*) *vs.* $P_{Nmax}$, $V_{cmax}$ or $J_{max}$ (taxa and $O_3$ treatments pooled). The same analysis ($n = 16$) was also conducted between the whole-plant dry matter and $P_{Nmax}$, $V_{cmax}$, and $J_{max}$, respectively, of Japanese larch ($O_3$ treatments pooled). Values of $R$ within the arbitrary segments [0.00, 0.10), [0.10, 0.30), [0.30–0.50), [0.50–0.70), [0.70–0.90), and 0.90+, can be translated to trivial, low, moderate, large, very large, and nearly perfect correlation magnitude, respectively (Hopkins 2000).

*MS EXCEL 2010* (*Microsoft* ©) and *STATISTICA v. 10* (*StatSoft Inc*. ©) software were used for data processing and statistics.

## Results

**Growth and dry matter**: NF60 significantly inhibited the RGR of plant height (Table 1) and stem basal diameter (Table 2) in both larches independently from time. Independently from taxa and $O_3$, RGR of height and diameter was increasing up to the second time period, decreasing from the third time period onwards to the fifth time period when it increased again. Taxa and the interaction $O_3 \times$ taxa were insignificant, but the interaction taxa $\times$ time was significant for the RGR of plant height and stem basal diameter. HL had greater height RGR over the first (226%) and second (128%) time periods, but lower over the third time period (45%) than that of JL in 2013; both larches had statistically indifferent height RGR over the fourth and fifth time periods in 2014 (grand means, Table 1). HL had also greater or lower diameter RGR over the first or third time periods, respectively, than that of JL; HL and JL had statistically indifferent diameter RGR over the second, fourth, and fifth time periods (grand means, Table 2). The interaction of $O_3 \times$ taxa $\times$ time was significant for height RGR (Table 1), but insignificant for diameter RGR (Table 2). In CF and NF40, HL had greater height RGR over the first and second time periods, lower over the third time period and indifferent over the fourth and fifth time periods, compared with JL (Table 1). In NF and NF60, HL had higher height RGR over the first time period, lower over the third time period, and indifferent over the second, fourth, and fifth time periods, compared to JL.

Plants after NF60 had significantly lower height gain than plants treated by CF, NF, and NF40, independently from taxa (Fig. 1*F*). HL had greater plant height than that of JL, independently from $O_3$ treatment. The interaction $O_3 \times$ taxa was insignificant (Fig. 1*F*).

Ozone treatments significantly affected the dry matter production of needles, branches, stem, roots, and of total plant (Fig. 1). HL had significantly lower needle dry matter (Fig. 1*A*) and greater stem dry matter (Fig. 1*B*) than that of JL, but branch (Fig. 1*D*), roots (Fig. 1*E*), and whole-plant dry matter (Fig. 1*C*) were not statistically different. The taxa performance varied significantly among $O_3$ treatments ($O_3 \times$ taxa) in dry matter of needles, branch, stem, and whole plant (Fig. 1*A–D*). HL had greater dry matter production of needles, branch, stem, and whole plant in CF treatment. However, HL had lower dry matter of needles than that of JL (Fig. 1*A*), but not statistically different dry matter of branch, stem, and whole plant (Fig. 1*B,C,D*) after the NF treatment. HL did not have also statistically different dry matter of needles, branch, stem, and whole plant from JL in NF40 and NF60 treatments (Fig. 1*A–D*). Dry matter of needles, branch, stem, and whole plant were reduced by NF, NF40, and NF60 treatments in HL, albeit it was not statistically significant for the stem. There was a decrease in the dry matter of needles, branch, stem, and whole plant of JL in NF60 treatment, whereas there was an increase in NF, compared with CF control, which was statistically insignificant.

**Leaf gas exchange**: Ozone was significant predictor in all gas-exchange parameters ($P_N$, $g_s$, $E$, $V_{cmax}$, $J_{max}$, and $P_{Nmax}$) for both HL and JL (Fig. 2). The NF treatment did not statistically differ from CF treatment in $P_N$, $g_s$, and $E$ (Fig. 2*A,B,D*); however, the NF treatment resulted in greater $V_{cmax}$, $J_{max}$, and $P_{Nmax}$ (Fig. 2*C,E,F*), compared with CF, for both HL and JL. The NF40 treatment suppressed $P_N$ and $g_s$ (Fig. 2*A,D*), but not $E$, $V_{cmax}$, $J_{max}$, and $P_{Nmax}$ (Fig. 2*B,C,E,F*), compared with CF. Similarly, the NF60 treatment suppressed all the gas-exchange parameters ($P_N$, $g_s$, $E$, $V_{cmax}$, and $P_{Nmax}$) except $J_{max}$ (Fig. 2*C*), compared with CF.

HL showed lower $P_N$, $g_s$, and $E$ than that of JL (Fig. 2*A,B,D*); $V_{cmax}$, $J_{max}$, and $P_{Nmax}$ did not significantly differ between taxa (Fig. 2*C,E,F*).

Table 1. Time course (mean ± SE) of relative growth rate (RGR) of plant height of Japanese larch and hybrid larch $F_1$ plants exposed to charcoal-filtered air (CF), nonfiltered air (NF), NF enriched with $O_3$ to reach 40 nmol mol$^{-1}$ (NF40) or NF enriched with $O_3$ to reach 60 nmol mol$^{-1}$ (NF60). RGR was calculated for the periods 17 June 2013 – 1 August 2013 (Time 1), 1 August 2013 – 6 September 2013 (Time 2), 6 September 2013 – 6 October 2013 (Time 3), 6 October 2013 – 15 June 2014 (Time 4), and 15 June 2014 – 20 September 2014 (Time 5). Different *uppercase letters* above time points or $O_3$ treatments show statistically significant difference between time points or $O_3$ treatments (within Time or $O_3$). Different *uppercase letters* above values of Time grand means of the two larches show statistically significant differences within the interaction taxa × time. Different *lowercase letters* above values show statistically significant differences within the interaction $O_3$ × taxa × time. Differences are marked according to HSD test. Each single mean is the average of four values, whereas each grand mean is the average of 16 values.

| Height RGR [cm cm$^{-1}$ d$^{-1}$] | | Time 1[A] | Time 2[D] | Time 3[A] | Time 4[B] | Time 5[C] |
|---|---|---|---|---|---|---|
| Hybrid larch | CF[A] | 0.00804 ± 0.00026[ijk] | 0.01519 ± 0.00096[n] | 0.00452 ± 0.00028[cdefg] | 0.00125 ± 0.00009[a] | 0.00686 ± 0.00024[fghijk] |
| | NF[A] | 0.00982 ± 0.00112[jklm] | 0.01324 ± 0.00073[mn] | 0.00463 ± 0.00068[cdefg] | 0.00112 ± 0.00016[a] | 0.00654 ± 0.00044[efghij] |
| | NF40[A] | 0.00966 ± 0.00056[iklm] | 0.01433 ± 0.00048[n] | 0.00311 ± 0.00034[bcd] | 0.00112 ± 0.00016[a] | 0.00721 ± 0.00035[ghijk] |
| | NF60[B] | 0.00725 ± 0.00065[ghik] | 0.00895 ± 0.00108[jk] | 0.00253 ± 0.00037[abc] | 0.00107 ± 0.00005[a] | 0.00694 ± 0.00052[fghijk] |
| | Grand mean | 0.00870 ± 0.00043[BE] | 0.01293 ± 0.00073[F] | 0.00370 ± 0.00031[D] | 0.00114 ± 0.00005[C] | 0.00689 ± 0.00019[A] |
| Japanese larch | CF[A] | 0.00461 ± 0.00029[cdefgh] | 0.01013 ± 0.00075[klm] | 0.00911 ± 0.00033[jkl] | 0.00140 ± 0.00013[ab] | 0.00778 ± 0.00023[ijk] |
| | NF[A] | 0.00382 ± 0.00046[cde] | 0.01310 ± 0.00080[mn] | 0.00907 ± 0.00126[jk] | 0.00133 ± 0.00012[ab] | 0.00758 ± 0.00025[hijk] |
| | NF40[A] | 0.00416 ± 0.00047[cdef] | 0.00892 ± 0.00086[jk] | 0.00877 ± 0.00089[ijk] | 0.00132 ± 0.00008[ab] | 0.00813 ± 0.00037[ijk] |
| | NF60[B] | 0.00282 ± 0.00034[abc] | 0.00828 ± 0.00089[ijk] | 0.00565 ± 0.0097[defghi] | 0.00145 ± 0.00007[ab] | 0.00766 ± 0.00042[ijk] |
| | Grand mean | 0.00385 ± 0.00025[D] | 0.01011 ± 0.00060[E] | 0.00815 ± 0.00056[AB] | 0.00137 ± 0.00005[C] | 0.00779 ± 0.00016[AB] |

repeated GLM test

| $O_3$ | Taxa | Time | $O_3$ × Taxa | $O_3$ × Time | Taxa × Time | $O_3$ × Taxa × Time |
|---|---|---|---|---|---|---|
| $P<0.001$ | $P>0.05$ | $P<0.001$ | $P>0.05$ | $P<0.001$ | $P<0.001$ | $P<0.01$ |

The interaction of $O_3$ × taxa was significant for $P_N$, $g_s$, and $E$ (Fig. 2A,B,D), while insignificant for $V_{cmax}$, $J_{max}$, and $P_{Nmax}$ (Fig. 2C,E,F). Regarding $P_N$, the only significant difference between larches within each $O_3$ condition was a greater mean of JL than HL in NF60 (Fig. 2A). However, while HL had not statistically different $g_s$ between $O_3$ treatments, JL showed lower $g_s$ in NF40 and NF60 compared with CF (Fig. 2D). Similarly, NF, NF40, and NF60 treatments did not significantly alter $E$ of HL compared with CF, but NF40 and NF60 suppressed $E$ of JL (Fig. 2B).

WUE did not differ between taxa or between $O_3$ treatments, and the interaction $O_3$ × taxa was also insignificant (Fig. 3D).

Regression analysis revealed a significant linear correlation, across all the treatments, of the whole-plant dry matter with $P_{Nmax}$ ($R^2=0.608$), $V_{cmax}$ ($R^2=0.727$), and $J_{max}$ ($R^2=0.706$), respectively, in JL.

**Leaf Chl and nutrients**: Ozone was significant predictor in all the contents [N, Chl *a*, Chl *b*, and Chl (*a+b*)], except C (Fig. 3). In comparison with CF, reductions were observed in the N content at NF40 and NF60 (Fig. 3A) and in the contents of Chl *a*, Chl *b*, and Chl (*a+b*) at NF60 (Fig. 3C,E,F).

HL had greater contents of C, Chl *a*, Chl *b*, and Chl (*a+b*) (Fig. 3B,C,E,F) than that of JL, whereas the N content did not differ between larches (Fig. 3A).

The interaction $O_3$ × taxa was significant only in the N content, which it was reduced by NF60 in HL and by NF40 and NF60 in JL, compared with CF (Fig. 3A). However, HL had 30% more Chl (*a+b*) in NF and NF40 than that in CF, but with large RSD and thus statistically insignificant; JL did not appear to have such a difference between CF and NF or NF40 (Fig. 3F).

Table 2. Time course (mean ± SE) of relative growth rate (RGR) of stem basal diameter of Japanese larch and hybrid larch $F_1$ plants exposed to charcoal-filtered air (CF), nonfiltered air (NF), NF enriched with $O_3$ to reach 40 nmol mol$^{-1}$ (NF40) or NF enriched with $O_3$ to reach 60 nmol mol$^{-1}$ (NF60). RGR was calculated for five time periods (*see* caption of Table 1). Different *uppercase letters* above time points or $O_3$ treatments show statistically significant difference between time points or $O_3$ treatments (within Time or $O_3$). Different *uppercase letters* above values of Time grand means of the two larches show statistically significant differences within the interaction taxa × time. Differences are marked according to HSD test. Each single mean is the average of four values, whereas each grand mean is the average of 16 values.

| Diameter RGR [mm mm$^{-1}$ d$^{-1}$] | | Time 1[C] | Time 2[E] | Time 3[D] | Time 4[A] | Time 5[B] |
|---|---|---|---|---|---|---|
| Hybrid larch | CF[A] | 0.00863 ± 0.00032 | 0.01487 ± 0.00052 | 0.00845 ± 0.00088 | 0.00178 ± 0.00019 | 0.00342 ± 0.00008 |
| | NF[A] | 0.00983 ± 0.00022 | 0.01560 ± 0.00057 | 0.00736 ± 0.00057 | 0.00181 ± 0.00010 | 0.00309 ± 0.00012 |
| | NF40[A] | 0.00848 ± 0.00067 | 0.01406 ± 0.00082 | 0.00619 ± 0.00068 | 0.00168 ± 0.00022 | 0.00382 ± 0.00020 |
| | NF60[B] | 0.00692 ± 0.00085 | 0.01035 ± 0.00114 | 0.00757 ± 0.00070 | 0.00179 ± 0.00013 | 0.00329 ± 0.00031 |
| | Grand mean | 0.00847 ± 0.00148[CD] | 0.01372 ± 0.00253[E] | 0.00739 ± 0.00153[C] | 0.00177 ± 0.00031[A] | 0.00341 ± 0.00045[B] |
| Japanese larch | CF[A] | 0.00508 ± 0.00021 | 0.01474 ± 0.00165 | 0.01138 ± 0.00087 | 0.00196 ± 0.00010 | 0.00299 ± 0.00027 |
| | NF[A] | 0.00690 ± 0.00073 | 0.01610 ± 0.00140 | 0.00964 ± 0.00110 | 0.00198 ± 0.00022 | 0.00266 ± 0.00020 |
| | NF40[A] | 0.00628 ± 0.00078 | 0.01218 ± 0.00091 | 0.01083 ± 0.00049 | 0.00194 ± 0.00013 | 0.00279 ± 0.00026 |
| | NF60[B] | 0.00350 ± 0.00052 | 0.01106 ± 0.00097 | 0.00872 ± 0.00155 | 0.00192 ± 0.00016 | 0.00331 ± 0.00036 |
| | Grand mean | 0.00544 ± 0.00172[F] | 0.01352 ± 0.00307[E] | 0.01014 ± 0.00220[D] | 0.00195 ± 0.00022[A] | 0.00294 ± 0.00056[B] |

| Repeated GLM test | | | | | | |
|---|---|---|---|---|---|---|
| $O_3$ | Taxa | Time | $O_3$ × Taxa | $O_3$ × Time | Taxa × Time | $O_3$ × Taxa × Time |
| $P<0.001$ | $P>0.05$ | $P<0.001$ | $P>0.05$ | $P<0.001$ | $P<0.001$ | $P>0.05$ |

Chl contents could effectively predict scores ($P<0.05$, adjusted $R^2<R^2$) on $P_{Nmax}$, $J_{max}$, and $V_{cmax}$ (Table 3). Chl *a* had a large correlation magnitude with $P_{Nmax}$ and a very large correlation magnitude with $J_{max}$ and $V_{cmax}$. Chl *b* and Chl (*a*+*b*) always had a large correlation magnitude with $P_{Nmax}$, $J_{max}$, and $V_{cmax}$.

## Discussion

Hormesis was clearly observed in $P_{Nmax}$, $J_{max}$, and $V_{cmax}$, and the quantitative characteristics of hormesis were similar for both larches. Hormesis may occur after a direct $O_3$-induced stimulation or through overcompensation after an $O_3$-induced disruption in homeostasis (Calabrese 2014, 2015). Plant stress in NF, as pictured by $P_{Nmax}$, $J_{max}$, and $V_{cmax}$, indicates disruption in the homeostasis, an unstable equilibrium in the physiological functioning (Agathokleous 2017).

Growth of Japanese larch and its hybrid was inhibited at NF60 but not at NF40 and NF. In NF60, $O_3$ stress was high enough to inhibit the competitive capacity of HL to the point that plant height was similar between HL and JL, consequently creating a similar light environment for both larches. Previous experiments also revealed a growth inhibition of both larches at approximately 60 nmol($O_3$) mol$^{-1}$

(Gyu *et al.* 2015, Agathokleous *et al.* 2017). The sensitivity of *Larix* spp. to elevated $O_3$ has been widely shown (Yamaguchi *et al.* 2011, Koike *et al.* 2012; Agathokleous *et al.* 2017). This sensitivity may relate to leaf habit as deciduous trees are considered to have higher $O_3$ sensitivity than evergreen trees (Paoletti 2006, Calatayud *et al.* 2010). Both larches shared a similar response to $O_3$ as shown by plant height and stem basal diameter, in agreement with earlier findings in a free air $O_3$-enrichment system (Agathokleous *et al.* 2017). However, greater initial height RGR of HL indicated higher competitiveness for light resources over the JL. This superiority of HL in height RGR lasted until autumn of the first growing season (6 September, 2013). In contrast to stem growth, height growth is one of the desirable characteristics of this hybrid which outperforms Japanese larch (Kurinobu 2005).
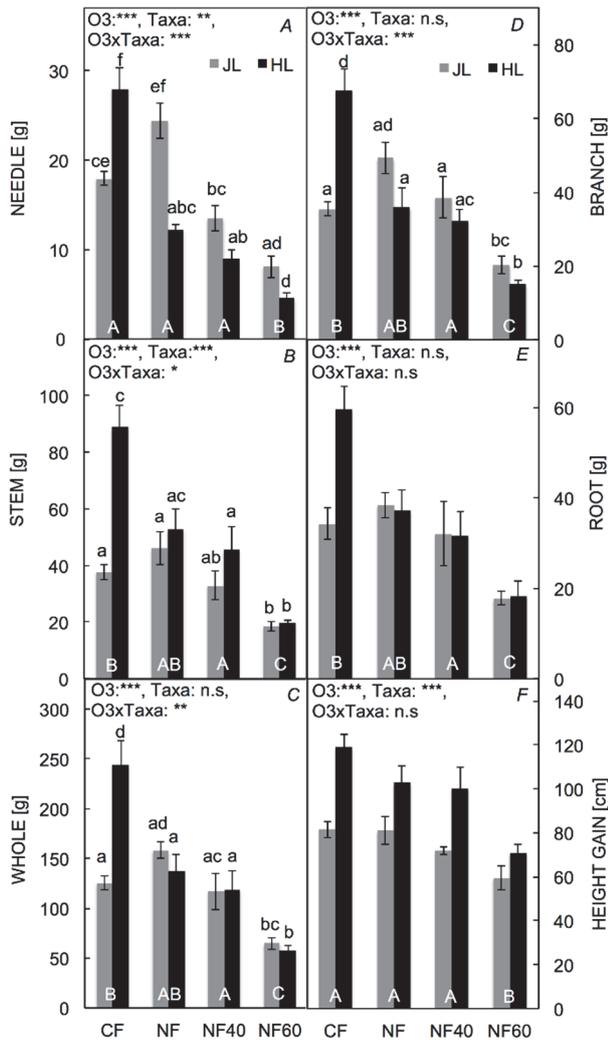
Fig. 1. Mean values ± SE ($n$ = 4) of dry matters and height gain of Japanese larch (JL) and hybrid larch $F_1$ (HL) exposed to charcoal-filtered air (CF), nonfiltered air (NF), NF enriched with O₃ to reach 40 nmol mol⁻¹ (NF40) or NF enriched with O₃ to reach 60 nmol mol⁻¹ (NF60). Asterisks indicate GLM significant effects at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***), whereas "n.s" indicates insignificant GLM effects ($P>0.05$). Different *uppercase letters* A, B, and C show statistically significant differences between the O₃ treatments (taxa pooled). Different *lowercase letters* above the means show statistically significant differences within the interaction O₃ × taxa. Differences are marked according to HSD test.

Insignificant interaction of O₃ × taxa in height RGR and height gain suggests that HL had an overall greater height than JL, indicating sustained heterosis and competitiveness of HL across O₃ treatments, as shown before (Agathokleous *et al.* 2017). Overall, the results of height RGR and height gain suggest that HL had a faster initial growth which contributed in the continuous superiority in height over JL.

HL outperformed JL in dry matter production at CF. HL had 149% greater WUE than that of JL in CF, albeit
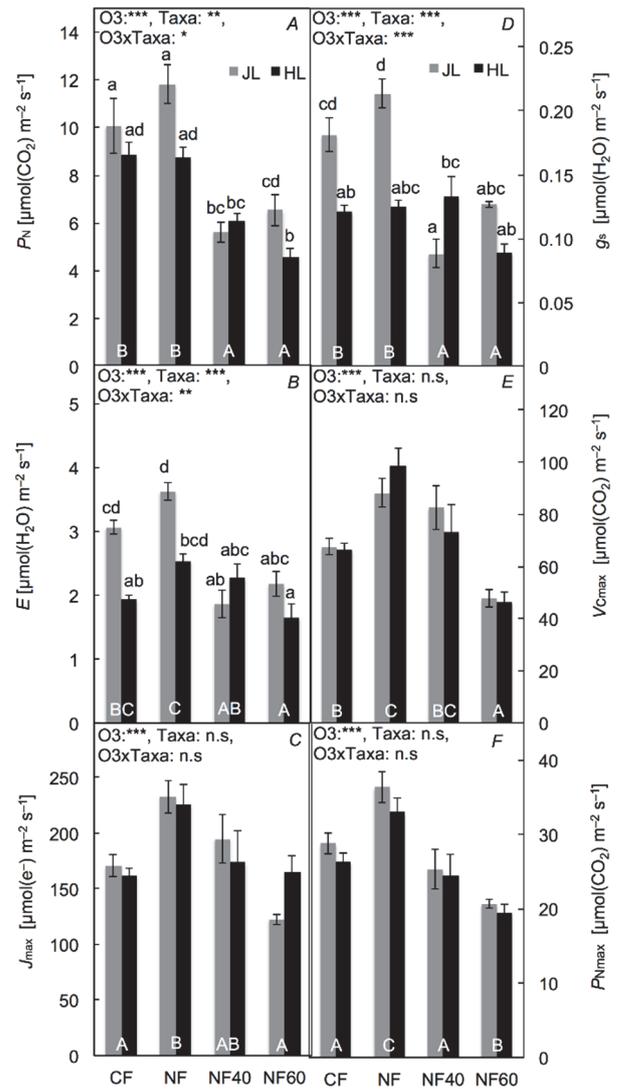


Fig. 2. Mean values ± SE ($n$ = 4) of gas-exchange parameters of Japanese larch (JL) and hybrid larch $F_1$ (HL) exposed to charcoal-filtered air (CF), nonfiltered air (NF), NF enriched with O₃ to reach 40 nmol mol⁻¹ (NF40) or NF enriched with O₃ to reach 60 nmol mol⁻¹ (NF60). The analyzed parameters are net photosynthetic rate ($P_N$), transpiration rate ($E$), and stomatal conductance ($g_s$) at light saturation and ambient $CO_2$ (380 μmol mol⁻¹), maximum photosynthetic rate at 1700 μmol($CO_2$) mol⁻¹ ($P_{Nmax}$), maximum rate of carboxylation ($V_{cmax}$), and maximum rate of electronic transport rate ($J_{max}$). Asterisks indicate GLM significant effects at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***), whereas "n.s" indicates insignificant GLM effects ($P>0.05$). Different *uppercase letters* A, B, and C show statistically significant differences between the O₃ treatments (taxa pooled). Different *lowercase letters* above the means show statistically significant differences within the interaction O₃ × taxa. Differences are marked according to HSD test.

the difference was statistically insignificant, which may explain the dry matter superiority of HL in CF. Despite the superiority of HL to JL in height gain, HL appeared to have greater O₃-induced reductions in dry matter than that of JL.
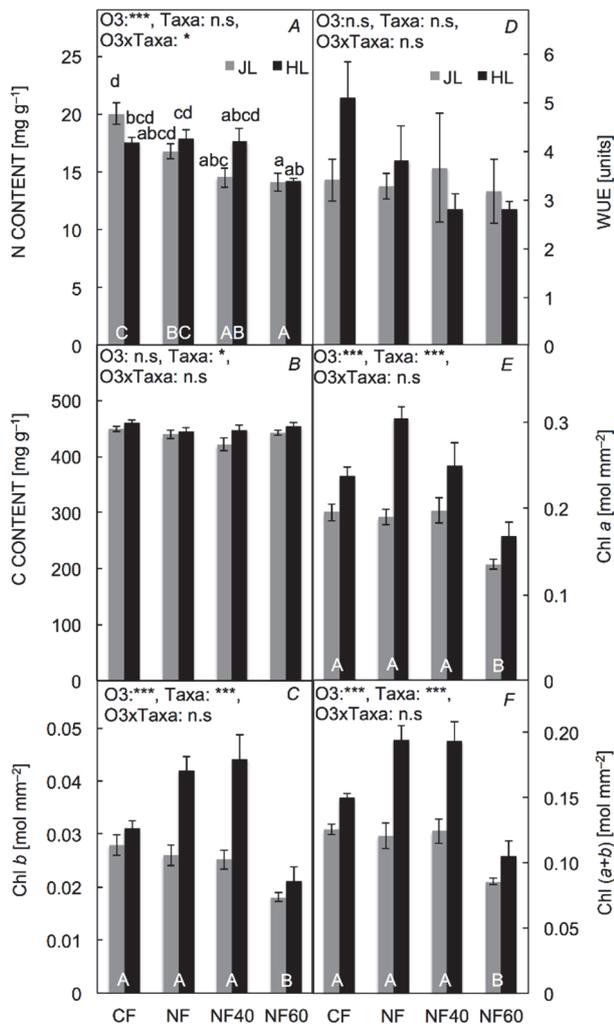
907

Fig. 3. Mean values ± SE (*n* = 4) of nitrogen (N), carbon (C), chlorophyll (Chl) *a*, Chl *b*, and Chl (*a+b*) contents and water-use efficiency (WUE) of Japanese larch (JL) and hybrid larch $F_1$ (HL) exposed to charcoal-filtered air (CF), nonfiltered air (NF), NF enriched with $O_3$ to reach 40 nmol $mol^{-1}$ (NF40) or NF enriched with $O_3$ to reach 60 nmol $mol^{-1}$ (NF60). Asterisks indicate GLM significant effects at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***), whereas "n.s" indicates insignificant GLM effects ($P>0.05$). Different *uppercase letters* A, B, and C show statistically significant differences between the $O_3$ treatments (taxa pooled). Different *lowercase letters* above the means show statistically significant differences within the interaction $O_3 \times$ taxa. Differences are marked according to HSD test.

HL had greater Chl contents than that of JL across $O_3$ treatments, likely because of greater amount of light reaching the foliage as HL grew taller than JL. Needle dry matter of JL was greater than that of HL in NF, but not in NF40 treatment. There are two potential explanations for the greater needle dry matter in JL compared to HL in NF: (*1*) accelerated leaf senescence in HL due to $O_3$ stress, a typical result of damaged Rubisco (Pell *et al.* 1994, Yendrek *et al.* 2013, Kitao *et al.* 2016); and (*2*) a difference in the period and timing of needle senescence and in the needle structure between the larches: needles usually fall faster in *L. gmelinii* than in *L. kaempferi* (*e.g.*, Ryu *et al.* 2009).

Photosynthesis was not limited by stomatal closure in NF, showing no activation of an $O_3$-avoidance mechanism. Rubisco activity was, nonetheless, upregulated by NF as indicated by an increase in $P_{Nmax}$, $V_{cmax}$, and $J_{max}$ in both taxa. This upregulation in $P_{Nmax}$ ($R^2=0.126$), $V_{cmax}$ ($R^2=0.178$), and $J_{max}$ ($R^2=0.026$) was not related to the measured N content in needles. It was, however, related to the Chl contents and, especially Chl *a* content, which was more effective than Chl *b* and Chl (*a+b*) in predicting the outcome of $P_{Nmax}$, $V_{cmax}$, and $J_{max}$. The upregulation in $P_{Nmax}$, $V_{cmax}$, and $J_{max}$ may suggest better performance of Rubisco, which is responsible for converting atmospheric $CO_2$. To our knowledge, this was the first evidence for upregulation of physiological performance in Rubisco by low $O_3$ mixing ratios. The effects may also relate to the antioxidant activity which affects the photosynthetic apparatus (Flowers *et al.* 2007). $V_{cmax}$ and $J_{max}$ were inhibited by NF60 in both larches, which might result in the decreased N content in NF60 compared to CF. The $O_3$ effects on photosynthetic reactions are defined by the amount of absorbed $O_3$ dose (Paoletti and Manning 2007). The greater $g_s$ in JL than that in HL in NF may suggest higher $O_3$ intake dose in JL. Hence, it can be postulated that the HL showed similar stress as JL, even if the $O_3$ intake dose in HL was lower than that in JL. Stress under NF seemed to lead to suppressed biomass productivity in HL but not in JL, a phenomenon which was not upon a higher $O_3$ intake dose in HL than in JL. Although statistically insignificant, the greater plant dry matter of JL at NF (+126%), compared to CF, was in agreement with greater $P_{Nmax}$ (+126%), $V_{cmax}$ (+130%), and $J_{max}$ (+136%). This was supported by the regression analysis which showed that increase (or decrease) in whole-plant dry

Table 3. Results of simple linear regression analysis between chlorophyll (Chl) contents and maximum photosynthetic rate at 1,700 $\mu$mol($CO_2$) $mol^{-1}$ ($P_{Nmax}$), maximum rate of carboxylation ($V_{cmax}$) or maximum rate of electronic transport rate ($J_{max}$), $n = 16$.

| | $P_{Nmax}$ | | $V_{cmax}$ | | $J_{max}$ | |
|---|---|---|---|---|---|---|
| Chl *a* | $R^2=0.436$ | $P<0.01$ | $R^2=0.540$ | $P<0.001$ | $R^2=0.529$ | $P<0.001$ |
| Chl *b* | $R^2=0.316$ | $P<0.05$ | $R^2=0.327$ | $P<0.05$ | $R^2=0.270$ | $P<0.05$ |
| Chl (*a+b*) | $R^2=0.400$ | $P<0.01$ | $R^2=0.442$ | $P<0.01$ | $R^2=0.416$ | $P<0.01$ |

matter was associated with an increase (or decrease) in $P_{Nmax}$, $V_{cmax}$, and $J_{max}$. Long-term experiments may be needed to depict the consequences of hormesis in productivity, because trees usually possess relatively high plasticity (*e.g.*, Küppers 1989).

Stimulation in gas-exchange traits by NF did not affect biomass at the whole plant level in JL. These observations suggest that stimulation of physiological functions, which occurs below the no observed adverse effect level (NOAEL) in hormetic exposure-response relationships, do not necessarily lead to benefits to the productivity at whole-plant level. Despite the physiological O₃-induced stimulation, it seems that HL could not sustain its productivity through plastic response to low O₃ exposures, whereas JL could do so. It should be mentioned that potential failure of plants to re-establish the disruption in the homeostasis in the appropriate time period can cause severe injuries. With reference to the C-S-R diagram of plant strategies (Grime 1977), when the larches were grown under low-exposure stress (*i.e.*, NF), HL seems to have a competitive strategy in this productive and relatively undisturbed vegetation, whereas JL seems to show a stress-tolerant strategy. The allocation of resources towards competitive capacity in HL along with the O₃ stress led to restricted productivity (*i.e.*, less dry matter) in

NF. In contrast, JL maintained the productivity in NF through stress-tolerant strategy. Such findings have been also reported by Liancourt *et al*. (2005) where the species competitive capacity was inverse to stress tolerance, *via* a trade-off between them.

**Conclusion**: Chronic exposure to O₃ induced hormetic response in both larches which showed stress at low and high O₃ exposures. Stress induced by low exposure to O₃ led to suppressed dry matter production in hybrid larch, but did not lead to lower dry matter in Japanese larch, suggesting a productive cost of O₃ stress in hybrid larch and a potential difference in the biological plasticity between larches.

The productive outcome under stress induced by low exposure to O₃ may be a function of the adaptive strategy followed by plants under simultaneous competition or disturbance. Further studies are needed to demonstrate whether the hormetic response is unequivocal and to better understand O₃-induced hormesis in trees. Attention should be paid to earlier belowground competition as it may influence the competition and thereby the effects of stress on productivity. Chl content and composition (Chl *a* and Chl *b*) may play an essential role in understanding O₃-induced hormesis in larches.

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